

# **pBK-CMV Phagemid Vector**

## **Instruction Manual**

**Catalog #212209**

Revision B

**Research Use Only. Not for Use in Diagnostic Procedures.**

212209-12



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# **pBK-CMV Phagemid Vectors**

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# pBK-CMV Phagemid Vector

## MATERIALS PROVIDED

Material provided	Quantity	Storage
pBK-CMV <sup>a</sup> phagemid vector(1 µg/µl)	20 µg	-20°C
XL1-Blue MRF <sup>b</sup>	500 µl	-80°C
R408 Interference-Resistant Helper Phage	1 ml	-80°C

<sup>a</sup> Supplied in 1 µg/µl in TE buffer [5 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA]

<sup>b</sup>  $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac$   
[F' proAB lac<sup>r</sup>ZΔM15 Tn10 (Tet<sup>r</sup>)]

## STORAGE CONDITIONS

**pBK-CMV Phagemid Vector:** -20°C

**Helper Phage:** -80°C

**Bacterial Glycerol Stock:** -80°C

## NOTICES TO PURCHASER

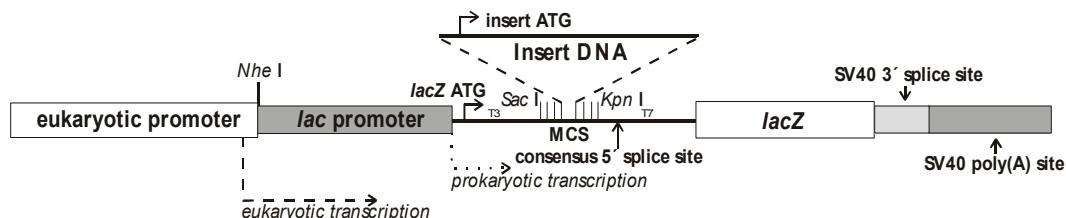
The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

## INTRODUCTION

The pBK-CMV phagemid vector<sup>1</sup> is a cloning vector derived from a high-copy-number pUC-based plasmid. This vector allows expression in both eukaryotic and prokaryotic systems. Eukaryotic expression is driven by the cytomegalovirus (CMV) immediate early promoter in the pBK-CMV phagemid vector. Stable clone selection in eukaryotic cells is made possible with G418 by the presence of the neomycin- and kanamycin-resistance gene, which is driven by the SV40 early promoter with thymidine kinase (TK) transcription termination and polyadenylation signals.<sup>2</sup> In the pBK-CMV phagemid vector, prokaryotic expression is driven by the *lac* promoter, which is repressed in the presence of the LacI protein and is inducible by IPTG. In bacteria expressing the *lacZΔM15* mutation and *lacI*, colonies containing vector without insert will be blue in the presence of X-gal and IPTG. Kanamycin-resistant colonies containing vector with insert will be white and can express the inserted gene as a fusion protein.

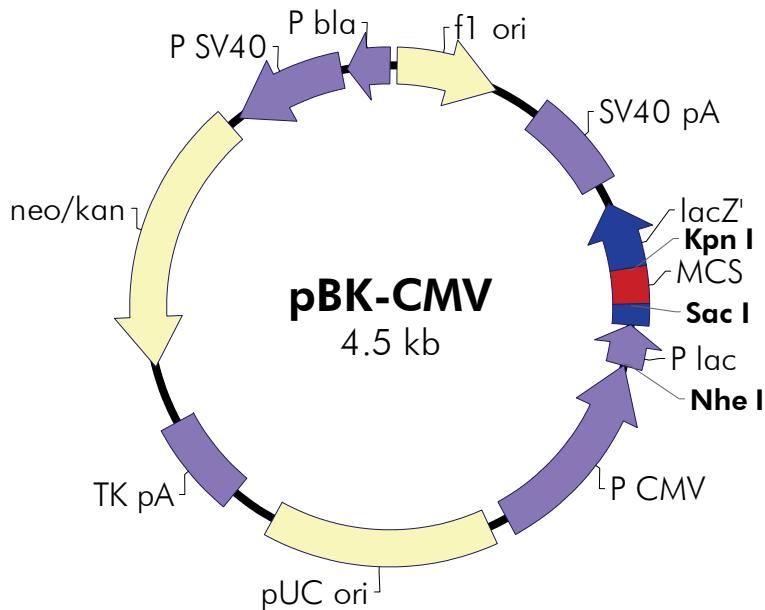
Inserts should be cloned within the polylinker cloning sites for prokaryotic expression. For eukaryotic expression one can clone either within the polylinker or between the *Nhe* I site at the 5' end of the *lac* promoter and a polylinker cloning site at the 3' end (see Fig. 1). Removing the upstream *lac* sequences from the eukaryotic transcript by cloning between the *Nhe* I site and the polylinker sites has been shown to give elevated eukaryotic expression with test inserts.

The pBK-CMV phagemid vector has an extensive polylinker in an SK orientation (the *Sac* I site is the closest restriction site to the *lacZ* promoter, and the *Kpn* I site is the farthest restriction site from the *lacZ* promoter). The polylinker contains 17 unique restriction enzyme recognition sites, organized with alternating 5' and 3' overhangs to allow serial exonuclease III/mung bean nuclease deletions.<sup>2</sup> Sites with compatible restriction overhangs, such as *Spe* I-*Xba* I and *Sal* I-*Xho* I, have been placed on opposite sides of the *Eco*R I site to allow unidirectional cloning in both the sense and antisense orientations with the ZAP Express vectors and the pBK-CMV phagemid vector. The (–) orientation of the f1 intergenic (IG) region in pBK-CMV allows rescue of antisense single-stranded DNA (ssDNA) relative to the *lacZ* transcript. This ssDNA can be used for dideoxynucleotide sequencing (Sanger method) or site-specific mutagenesis. Flanking the polylinker are T3 and T7 RNA polymerase promoters that can be used to synthesize RNA *in vitro*. The choice of promoter used to initiate transcription determines which strand of the DNA insert will be transcribed.



**Figure 1** Expression cassette in the pBK-CMV phagemid vector.

## The pBK-CMV Vector



### pBK-CMV Multiple Cloning Site Region (sequence shown 952–1196)

T7 Promoter →  
 GTAAAACGACGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGTACACTTACCTGGTACCCACCCGGGTGGAAA...  
 M13 –20 primer binding site      T7 primer binding site

...ATCGATGGGCCCGCGCCGCTCTAGAAGTACTCTCGAGAAGCTTTGAATTCTTGGATCCACTAGTGTGACCTGCAG...  
 Apa I      Not I      Xba I      Sca I      Xho I      Hind III      EcoR I      BamH I      Spe I      Acc I  
 BssH II      Sac I      ← T3 Promoter      ← β-gal α-fragment      ← BK reverse primer binding site  
 ...GCGCGCAGCTCCAGCTTTGTTCCCTTAGTGAGGGTTAATTTCGAGCTTGGCTAATCAAGGTCAAGCTGTTCTGT  
 T3 primer binding site

Feature	Nucleotide Position
f1 origin of ss-DNA replication	24–330
SV40 polyA signal	469–750
β-galactosidase α-fragment coding sequence (lacZ')	812–1183
multiple cloning site	1015–1122
lac promoter	1184–1305
CMV promoter	1306–1895
pUC origin of replication	1954–2621
HSV-thymidine kinase (TK) polyA signal	2760–3031
neomycin/kanamycin resistance ORF	3209–4000
SV40 promoter	4035–4373
bla promoter	4392–4518

**FIGURE 2** The pBK-CMV phagemid vector. The complete sequence and list of restriction sites are available at [www.genomics.agilent.com](http://www.genomics.agilent.com).

## TRANSFORMATION WITH THE pBK-CMV PHAGEMID VECTOR

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**Note** *The pBK-CMV phagemid vector will replicate autonomously as plasmid DNA. Therefore, colonies—not plaques—are obtained following transformation.*

### Suggested Host Strain and Genotype

The XL1-Blue MRF' host strain is recommended for propagation of pBK-CMV phagemids and for transformation of recombinant phagemids. XL1-Blue MRF' allows blue-white color selection and single-stranded DNA rescue, and is restriction-deficient aiding in the construction of libraries made from methylated DNA.<sup>3</sup>

**XL1-Blue MRF' Genotype:**  $\Delta(mcrA)183$   $\Delta(mcrCB-hsdSMR-mrr)173$   $endA1$   $supE44$   $thi-1$   $recA1$   $gyrA96$   $relA1$   $lac$  [F' *proAB lacI<sup>q</sup>ZΔM15* Tn10 (Tet<sup>r</sup>)]

**Note** *The XL1-Blue MRF' is provided as a glycerol stock. Additional tubes of glycerol stock are available for purchase (Catalog #200301). Alternatively, high-efficiency XL1-Blue MRF' frozen competent cells are also available ( $>1 \times 10^9$  colonies/ $\mu$ g of pUC 18, Catalog #200230).*

For the appropriate media and plates for growth of XL1-Blue MRF', please refer to the following table:

Bacterial strain	Plates for bacterial streak	Media for glycerol stock
XL1-Blue MRF'	LB-tetracycline agar <sup>a</sup>	LB-tetracycline <sup>a</sup>

<sup>a</sup>12.5  $\mu$ g/ml tetracycline.

### Streaking Cells from a -80°C Bacterial Glycerol Stock

Prepare the following from a frozen glycerol stock:

**Note** *Do not allow the contents of the vial to thaw. The vials can be stored at -20° or -80°C, but most strains remain viable longer if stored at -80°C.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.
2. Streak the splinters onto an LB plate containing the appropriate antibiotic.

Restreak the cells fresh each week.

## Preparation of a -80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of the appropriate liquid media with one or two colonies from a plate of freshly-streaked cells. Grow the cells to late log phase.
2. Add 4.5 ml of a sterile glycerol-liquid media solution (prepared by combining 5 ml of glycerol + 5 ml of liquid media) to the bacterial culture from step 1. Mix well.
3. Aliquot into sterile centrifuge tubes (1 ml/ tube). This preparation may be stored at -20°C for 1-2 years or at -80°C for more than 2 years.

## Blue-White Color Selection

The XL1-Blue MRF' strain allows blue-white color selection for the pBK-CMV phagemid because of *lacZΔM15* complementation on the F' episome. The color selection may be seen when plating on LB plates containing 50 µg/ml of kanamycin, 80 µg/ml of fresh X-gal, and 20 mM IPTG. Alternatively, plates for color selection can be prepared by spreading 100 µl of 40 mM IPTG and 100 µl of 2% X-gal on LB-kanamycin agar plates 30 minutes prior to plating your transformants. X-gal should be prepared in dimethyl formamide and IPTG prepared in sterile, distilled H<sub>2</sub>O (store stock solutions at -20°C until use). Colonies containing phagemids without inserts will be blue after incubation for 12-18 hours at 37°C. Colonies with phagemids containing inserts will remain white. Further enhancement of the blue color may be obtained by placing plates at 4°C for 2 hours following overnight growth at 37°C.

Occasionally, β-galactosidase fusion proteins are toxic to the host bacteria. If there is any suspicion that an insert might be toxic, the X-gal and IPTG may be left out of the LB-kanamycin agar plates. Under these conditions there will be no color selection, but recombinants will express lower levels of the potentially toxic proteins.

## Background White Colonies

The *lacZΔM15* gene carried on the F' episome is needed for the blue-white color assay; therefore, host bacteria that have lost the F' episome will remain as white colonies on an agar plate containing X-gal and IPTG, even if the pBK-CMV phagemid vector does not contain an insert. XL1-Blue MRF' is a *lac*<sup>-</sup> AG1 derivative with *Tn10*, *lacI<sup>q</sup>* and *lacZΔM15* on the F' episome. Selection for bacteria containing the F' episome in this strain is accomplished by plating on LB-tetracycline agar plates. XL1-Blue MRF' transformants containing pBK-CMV phagemid vectors can be plated on LB-tetracycline-kanamycin agar plates<sup>8</sup> to select for colonies that contain both the F' episome and the pBK-CMV phagemid vector. This double selection further reduces the background of false positives.

For bacteria containing an F' episome without a *Tn10* gene, growth on a minimal medium plate supplemented with 1 mM thiamine-HCl will maintain selection for the F' episome; however, colonies will grow more

slowly (the JM109 strain appears 'slimy' on minimal plates). If it is questionable whether a JM109 white colony represents a pBK-CMV recombinant or an F<sup>-</sup> clone, streak the colony onto a minimal medium plate such as M9 media.<sup>§,3</sup> An F<sup>-</sup> cell will not grow; an F<sup>+</sup> cell will grow slowly because it carries the *proAB* genes on the F' episome.

## Ligation into the pBK-CMV Phagemid Vector

**Note** *We have observed that using excess amounts of EcoR I to digest pBK-CMV results in EcoR I prime activity. This appears as cleavage at a non-EcoR I site at the 3' end of the f1 intergenic region, causing confusion when interpreting results from an agarose gel. If a restriction pattern appears incorrect, use Optimal Buffer #7<sup>§</sup> for the digestion reaction and check whether reducing the units of EcoR I restores a normal restriction pattern.*

Dephosphorylate the digested pBK-CMV phagemid with calf intestinal alkaline phosphatase (CIAP) prior to ligation with the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the digested vector DNA on an agarose gel and recovering the desired vector band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.

After purification and ethanol precipitation of the restricted DNA, resuspend it in a volume of 5 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA that will allow the concentration of the vector DNA to be ~0.1 µg/µl (~0.07 pmol ends/µl).

For ligation, the ideal ratio of insert-to-vector DNA varies; however, a reasonable starting point is 2:1 (insert-to-vector ratio), measured in available picomole ends. Determine this starting point using the following calculation:

$$\text{picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

We suggest the following protocol which includes three controls:

Sample	1	2	3	4	5
Prepared vector (0.1 µg/µl)	1 µl	1 µl	1 µl	1 µl	0 µl
Prepared insert (0.07 pmol ends/µl)	2 µl	3 µl	0 µl	0 µl	1 µl
10 mM rATP (pH 7.0)	1 µl				
10× ligase buffer <sup>a</sup>	1 µl				
Double-distilled water (ddH <sub>2</sub> O)	4.5 µl	3.5 µl	6.5 µl	7.0 µl	6.5 µl
T4 DNA ligase (4 Weiss U/µl)	0.5 µl	0.5 µl	0.5 µl	0 µl	0.5 µl

<sup>§</sup> See Preparation of Media and Reagents.

1. Ligate for 2 hours at room temperature (22°C) or overnight at 4°C. When ligating blunt ends, ligate overnight at 12–14°C.
2. Transform 1–2  $\mu$ l of the ligation mixture into the appropriate competent bacteria. Plate on selective media.
3. Interpretation of test results:
  - ◆ Samples 1 and 2 vary the insert-to-vector ratio.
  - ◆ Sample 3 tests for the effectiveness of the CIAP treatment.
  - ◆ Sample 4 indicates if the vector was cleaved completely or if residual uncut vector remains.
  - ◆ Sample 5 verifies that the insert alone is not contaminated with uncut vector DNA.

Expected test results:

- ◆ Plates 1 and 2 should have mostly white colonies, representing recombinants.
- ◆ Plate 3 should have low numbers of blue colonies if the CIAP treatment was effective.
- ◆ Plate 4 should have no colonies if the digest was complete.
- ◆ Plate 5 should have no colonies if the insert was pure.

## EUKARYOTIC SCREENING

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Screening libraries in eukaryotic cells has proved to be a very effective way of identifying clones that are not possible to identify using prokaryotic screening systems. The screening technique used will depend on the clone of interest and on the type of assay available. An appropriate cell line for screening must be obtained, and an assay or reagent capable of identifying the cell or cells expressing the desired target protein must be developed. Three different techniques are available: selection, panning and functional analysis of clone pools.

For elevated eukaryotic expression of full-length cDNA inserts (i.e., clones containing their own ATG and Kozak sequence), the inserts can be cloned between the *Nhe* I site at the 5' end and any site in the polylinker at the 3' end. For some inserts, removal of the *lac* promoter and *lacZ* ATG results in increased expression (in-house observation). Non full-length cDNAs should be cloned within the polylinker, since these cDNAs require the *lacZ* ATG for expression of fusion proteins.

Clones identified by DNA hybridization or prokaryotic expression screening can be converted for increased eukaryotic expression by digesting with *Nhe* I and *Spe* I. These enzymes give compatible sticky ends. The digested DNA can then be ligated directly, transformed into competent *Escherichia coli* cells and screened for removal of the *lac* promoter by the loss of upstream polylinker sites (e.g., *Sac* I, *Bss*H II, *Pst* I and *Sal* I) and reduced phagemid size (202 bp). If *Nhe* I or *Spe* I sites exist within the insert sequences, different cloning procedures can be used to remove the upstream *lac* sequences from the 5' UT region.

### Selective Assay

Devising a selective assay for eukaryotic library screening requires a cell line that can grow in nonselective media and requires the expression of an additional protein for proliferation when grown in selective media. An example of this method is screening for a TK gene in L-TK<sup>-</sup> cells. If TK<sup>-</sup> cells are grown in HAT media, only those cells transfected with a clone coding for a protein capable of replacing TK will grow.

### Panning Assay

Clone identification by "panning" requires the transfection of a library into a cell line deficient in the desired surface protein. The clone of interest is expressed on the surface of eukaryotic cells, making the translated protein product accessible to an antibody, ligand or receptor coupled either directly or indirectly to a solid-phase matrix. Eukaryotic transfectant clones expressing the appropriate insert will bind to the affinity matrix, while cells not adhering are washed away. Either transient or stable transfection protocols can be used. Panning by transient transfection requires returning to the original DNA pool and screening successively smaller pools of clones until the desired clone is isolated. When panning stable transformants, positive pools are split into successively fewer cells per tissue culture well until the positive clones are isolated.

## Functional Assay

Functional assay screening can also be performed on either transient or stably transfected cells.<sup>4</sup> Transient expression will likely require subdividing the amplified library into smaller pools of clones to prevent the dilution of a positive cell signal with an excess of negative clones. Each clone pool is amplified separately and transfected into the eukaryotic cells. The transfected cells are then tested for the expression of the desired clone. Once a pool is identified as containing the clone of interest, the pool is subdivided into smaller pools for a second round of prokaryotic amplification, eukaryotic transfection and screening. After several rounds of enriching for the desired clone, a single clone can be isolated. The initial pool size is determined according to the sensitivity of the available assay so that a single clone within the pool is still theoretically detectable in the transfected cells. For example, if a positive assay signal is 1000-fold above background, pools containing 500–1000 members should still give a signal above background. The sensitivity of the assay dictates the initial size of the pools as well as the number of pools required to screen. If stable transformants are created using G418 selection, pools of stable clones can be assayed. This simplifies the identification of isolated positive eukaryotic clones, because the eukaryotic colonies can be picked or diluted in microtiter tissue culture plates.

Decreasing the initial library size when screening in eukaryotic cells reduces the number of background clones and the number of pools. Creating subtraction libraries can significantly reduce the primary library size by removing clones not specific to the source of the desired clone.<sup>5</sup> If the amino-terminal sequence of the desired protein is known, or if the clone is suspected to belong to a member of a conserved family, PCR amplification can be used to create an enriched library.

After a clone has been identified within the eukaryotic cells, the clone can be retrieved by several methods. Plasmid DNA within the tissue culture cells can be collected using the Hirt procedure, then transferred into *E. coli* cells for amplification and plasmid DNA preparations.<sup>6, 7</sup> Simmons *et al.* were able to screen libraries in COS cells, where the presence of the SV40 T antigen increases the copy number of phagemids containing the SV40 origin of replication.<sup>8</sup> This results in a higher episomal copy number which may help in the retrieval of the plasmids. Inserts can also be identified by PCR amplification of the tissue culture cells using T3/T7 primer sets. The resulting PCR fragment can be digested using restriction sites flanking the insert, then recloned into pBK-CMV phagemid DNA for further analysis.

## Protocol for Pool Amplification in the pBK-CMV Phagemid Vector

1. Plate the desired number of colonies on LB–kanamycin agar plates (50 µg/ml) and grow to ~1 mm in diameter.
2. Pool the colonies from each plate in 5 ml of LB–kanamycin broth (50 µg/ml) (see *Preparation of Media and Reagents*).
3. Grow a portion of each pool in the desired volume of liquid culture overnight at 37°C.

4. Prepare the plasmid DNA for each pool by cesium gradient banding or an equivalent protocol to obtain transfection-grade plasmid DNA.<sup>3</sup>
5. Transfect the plasmid DNA into the eukaryotic cells and test for stable or transient expression.
6. After the pool (containing the clone of interest) has been identified, transform fresh *E. coli* cells with the plasmid DNA from the primary pool and plate fewer colonies per plate than in step 1 above. Pool the colonies from each plate and prepare the plasmid DNA separately from each of these smaller pools, as described in steps 1–4 above.
7. Screen the DNA for eukaryotic expression. Make the pools successively smaller until a single positive clone can be identified.

## PROKARYOTIC SCREENING

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Colonies containing the pBK–CMV phagemid vector may be screened as follows: for recombinants by double-stranded DNA (dsDNA), RNA or oligonucleotide hybridization;<sup>9</sup> by restriction mapping or by sequencing miniprep plasmid DNA; and for expression of the insert using specific antibodies.

When growing plasmid libraries, remember that the bacteria can produce fusion proteins that contain several amino acids from the amino-terminal end of the  $\beta$ -galactosidase protein (3.5 kDa to the *Eco*R I site). Some fusion proteins are toxic to *E. coli*, and the colony may be lost from the library if the insert is expressed. Therefore, it is best to maintain plasmid libraries in the absence of IPTG, only inducing when screening for expression.<sup>10</sup>

### Prokaryotic Expression Screening Protocol

1. Plate transformants directly onto nitrocellulose membranes placed on LB–kanamycin agar plates (50  $\mu$ g/ml).
2. Grow the colonies at 37°C to 1 mm in diameter. (Do not exceed 1 mm in order to minimize background.) This nitrocellulose membrane will serve as the master membrane.
3. Pre-wet fresh nitrocellulose membranes by placing the membranes in 1 mM IPTG in water. Air-dry the membranes on Whatman® 3MM paper.
4. Remove the master nitrocellulose membrane from the LB–kanamycin agar plate (50  $\mu$ g/ml) and place the master membrane colony-side-up onto Whatman 3MM paper.
5. Carefully place a prewetted nitrocellulose membrane (from step 3 above) onto the master membrane. Overlay the two membranes with a second sheet of Whatman 3MM paper and apply even pressure.

6. Mark for orientation by inserting an 18-gauge needle through the two membranes in several different places. These holes will be used to align the master membrane with the replica membrane after screening the colonies.
7. Carefully peel the two membranes apart and place the replica membrane on an LB-kanamycin agar plate with the colonies facing upward. Repeat steps 5–7 with a second prewetted membrane from step 3 above. Grow the master membrane at 37°C on an LB-kanamycin agar plate (colony side up) for a few hours to let the colonies regenerate. Store the master membrane at 4°C on the LB-kanamycin agar plates.
8. Incubate the replicate membranes until their colonies grow to ~1 mm in diameter (do not exceed 1 mm in order to minimize background).
9. Proteins can be fixed to the membrane by chloroform lysis before screening.<sup>3</sup>

**Note** When screening with antibodies, the Agilent picoBlue immunoscreening kit is recommended.

10. To synthesize large quantities of the fusion protein in liquid culture, grow the cells to an OD<sub>600</sub> of 0.7 in the absence of IPTG. Then add IPTG to 1 mM and grow for an additional 2–3 hours.

## Colony Screening by DNA Hybridization

Recombinant clones derived from the pBK-CMV phagemid vector can be identified by colony hybridization. The following protocol minimizes problems associated with colony screening procedures. Perform the screening on **duplicate sets of membranes**.

## Fixing Replica Sets of Colonies onto Nitrocellulose Membranes

Use the following protocol to make multiple replica plates of transformants. Keep the original or master membrane to select colonies identified by the screening of the replica membranes.

1. Plate the transformants directly onto nitrocellulose membranes placed on LB-kanamycin agar plates (50 µg/ml).
2. Grow the colonies at 37°C to 1 mm in diameter. (Do not exceed 1 mm in order to minimize background.) This nitrocellulose membrane will serve as the master membrane.
3. Pre-wet fresh nitrocellulose membranes by placing the membranes on LB-kanamycin agar plates.
4. Remove the master nitrocellulose membrane from the LB-kanamycin agar plate (50 µg/ml) and place the master membrane colony side up onto Whatman 3MM paper.

5. Carefully place a prewetted nitrocellulose membrane from the LB-kanamycin agar plate in step 3 above onto the master membrane. Overlay the two membranes with a second sheet of Whatman 3MM paper and apply even pressure.
6. Mark for orientation by inserting an 18-gauge needle through the two membranes in several different places. These holes will be used to align the master membrane with the replica membrane after screening the colonies.
7. Carefully peel the two membranes apart and place the replica membrane on an LB-kanamycin agar plate with the colonies facing upward. Repeat steps 5–7 with a second prewetted membrane from step 3 above. Grow the master membrane at 37°C on an LB-kanamycin agar plate (colony side up) for a few hours to let the colonies regenerate. Store the master membrane at 4°C on the LB-kanamycin agar plates.
8. Incubate the replicate membranes until their colonies grow to ~1 mm in diameter (do not exceed 1 mm in order to minimize background).
9. Lyse the bacteria and denature the plasmid DNA on the nitrocellulose membranes using the following procedure:
  - a. Prepare three trays, each containing several sheets of Whatman 3MM paper. Pour enough of the following solutions into each tray to soak the membranes, but avoid excess fluid:

Tray 1	A 0.5 M NaOH solution
Tray 2	A 1.0 M Tris-HCl (pH 7.5) solution
Tray 3	A 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl solution
  - b. Remove the nitrocellulose membranes from the LB agar plates and place them on the wet Whatman 3MM paper, colony side up, proceeding as follows:

**Note** *The solution should soak through and wet the nitrocellulose membranes, using the Whatman 3MM paper as a wick. Never allow the solutions to cover the nitrocellulose membranes.*

Tray 1	Soak 5 minutes, then blot on dry Whatman 3mm paper for 5 minutes
Tray 2	Soak 2 minutes, then blot on dry Whatman 3mm paper for 2 minutes
Tray 3	Soak 10–15 minutes, then blot on dry Whatman 3mm paper for 2 minutes
  - c. Fix the DNA to the nitrocellulose membranes with the Stratalinker UV crosslinker using the autocrosslink setting (120,000 µJ of UV energy). Alternatively, place the membranes between pieces of Whatman 3MM paper and oven bake at 75–80°C for 1–1.5 hours.

At this stage, the nitrocellulose membranes are ready for hybridization and are stable indefinitely.

## Prehybridization of Southern Blots for Oligonucleotide Probes

1. Prehybridize the membrane in the following solution at 0.05–0.1 ml/cm<sup>2</sup> for 1–2 hours at 42°C with constant agitation in a heat-sealable hybridization bag:

6× SSC buffer<sup>§</sup>  
5× Denhardt's reagent<sup>§</sup>  
20 mM Na<sub>2</sub>PO<sub>4</sub>

500 µg/ml of denatured, sheared or sonicated salmon sperm DNA

## Hybridization of Oligonucleotide Probes to Southern Blots

**Notes** Prehybridize or hybridize the nitrocellulose membranes using the Agilent QuikHyb hybridization solution or follow the procedure outlined below.

During the prehybridization period, an oligonucleotide probe may be produced using T4 polynucleotide kinase (PNK) and high-specific-activity [ $\gamma$ -<sup>32</sup>P]ATP (>7000 Ci/mmol). Unincorporated radiolabeled nucleotides must be removed from the probe before hybridization. It is not necessary to denature oligonucleotide probes. An acceptable specific activity for an oligonucleotide probe is  $\geq 10^9$  cpm/µg.

1. Pour off the prehybridization solution.
2. Add the following hybridization solution to the nitrocellulose membranes (~0.05 ml/cm<sup>2</sup>):

6× SSC buffer  
20 mM Na<sub>2</sub>PO<sub>4</sub>  
0.4% (w/v) sodium dodecyl sulfate (SDS)<sup>¶</sup>  
500 µg/ml of denatured, sonicated salmon sperm DNA
3. Mix the labeled oligonucleotide probe with a minimum amount of hybridization solution and add this mixture to the hybridization bag containing the nitrocellulose membranes. The oligonucleotide probe concentration should be at least  $0.5 \times 10^6$  cpm/ml.
4. Massage the hybridization bag to evenly distribute the probe. Incubate for 12–18 hours at 42°C with constant agitation.

<sup>§</sup> See Preparation of Media and Reagents.

<sup>¶</sup> The SDS concentration can be increased to 1% (w/v) for Duralon-UV membranes and Illuminator nylon membranes.

## Posthybridization

1. Calculate the approximate melting temperature of the oligonucleotide probe as follows:<sup>3</sup>

**Note** *The first method below overestimates the  $T_m$  of hybrids involving longer oligonucleotides. The second formula works only for  $Na^+$  concentrations of  $\leq 1 M$ .*

### Oligonucleotides Shorter than 18 Bases

$$T_m = 2^\circ C(A + T) + 4^\circ C(G + C)$$

### Oligonucleotides 14 Bases and Longer (up to 60–70 Nucleotides)

$$T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) - (600/N)$$

where  $N$  is the chain length.

2. Pour the hybridization mix into a suitable container for liquid radioactive waste.
3. Wash the membrane at room temperature for 10 minutes in a 6× SSC buffer and 0.1% (w/v) SDS wash solution to remove unbound probe and hybridization solution.
4. Wash the membrane twice for 15 minutes each at a temperature 10°C below the calculated  $T_m$ .
5. Wash the membrane several times for 15 minutes each at 5°C below the calculated  $T_m$  in a 6× SSC buffer and 0.1% (w/v) SDS wash solution if necessary.
6. Blot excess solution off the membrane with blotting paper and seal the damp (not wet) membrane either in a heat-sealable hybridization bag or in plastic wrap. Expose the membrane to autoradiographic film at –80°C in an X-ray cassette with an intensifying screen. Exposure time is primarily determined by the amount of target DNA on the blot and the specific activity of the probe.

## Prehybridization and Hybridization Buffer for Double-Stranded Probes

**Note** *Prehybridize or hybridize the nitrocellulose membranes using the Agilent QuikHyb hybridization solution or follow the procedure outlined below.*

1. Make the appropriate amount of the following prehybridization–hybridization solution:

10 ml of 10× PIPES buffer  
12 ml of water  
25 ml of 100% deionized formamide  
2.5 ml of 10% (w/v) SDS  
0.5 ml of salmon sperm DNA (10 mg/ml)  
50 ml total volume

**Note** *Follow the same recipe for both the prehybridization and hybridization solutions.*

2. Plate and make duplicate sets of colony membranes as described in *Fixing Replica Sets of Colonies onto Nitrocellulose Membranes*.
3. Prehybridize the membrane in prehybridization solution at 0.05–0.1 ml/cm<sup>2</sup> for 1–2 hours at 42°C in a heat-sealable hybridization bag.
4. Prewarm fresh hybridization solution to 42°C. Boil the appropriate amount of salmon sperm DNA with the probe for 4 minutes in a volume <1 ml and add the salmon sperm DNA and the probe to the prewarmed hybridization solution. Decant the prehybridization solution from the hybridization bag and replace it with the hybridization solution and probe. Hybridize overnight at 42°C.
5. For long DNA probes (>50 bp), increase the stringency of the washes, starting with a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution at 55°C to wash the membranes. Wash the membranes three times for 15 minutes each.

## TRANSCRIPTION BY T3 AND T7 RNA POLYMERASE

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The RNA transcripts synthesized from inserts cloned into the pBK–CMV vector from either the T3 or the T7 polymerase promoter can be used for many purposes, including both Southern and Northern hybridization experiments and S1 or RNase A analysis. In addition, RNA transcripts can be used to produce protein by translation in vitro or translation in vivo after microinjection into *Xenopus* oocytes or tissue culture cells.

## Handling RNA

**Note** *Always wear gloves to prevent RNase contamination.*

When working with RNA, it is critical to prevent RNase contamination from any source. The following suggestions will help produce full-length transcripts:

- ♦ Make all buffers, dithiothreitol (DTT) and triphosphates in highly purified water, treated with diethylpyrocarbonate (DEPC). Add DEPC to water to a final concentration of 0.1%, heat to 37°C for 8 hours and autoclave. If the DEPC scent remains after autoclaving, place the water in a 90°C water bath until the scent is gone (~1 hour).

**Note** *Do not autoclave Tris solutions with DEPC!! Instead, use water that has been treated with DEPC to make all Tris solutions.*

- ♦ The Agilent RNAMaxx high yield transcription kit (Catalog #200339) is recommended for transcription reactions performed with T7 RNA polymerase, and contains enough reagents for 50 transcriptions, including T7 RNA polymerase.
- ♦ All tubes and pipet tips should be autoclaved and baked for several hours at 80°C. A common source of RNase contamination on gel electrophoresis equipment comes from miniprep DNA which has been treated with RNase A. Thoroughly clean all gel tanks, gel combs, gel spacers and glassware, using soap and water, followed by an ethanol rinse. Next soak the equipment in 3% hydrogen peroxide for 10 minutes at room temperature and rinse with DEPC-treated water. Keep cleaned items covered and away from bare hands. Autoclave all glass plates and other appropriate materials on the dry cycle before use.
- ♦ Phagemid templates for transcription must be RNase-free. Cesium chloride preps are advisable, but minipreps may be used if care is taken to remove contaminating RNases. Generally the plasmid template is linearized with an enzyme that cleaves "downstream" of the RNA polymerase promoter and the insert in the multiple cloning site (MCS). It is strongly advised to purify the post-restriction digest DNA by adding 50 µg/ml of proteinase K to the restriction reaction then incubate at 37°C for 30 minutes. Perform two phenol-chloroform [1:1 (v/v)] extractions and an ethanol precipitation, prior to the transcription reaction. Resuspend the digested, proteinase K-treated DNA at 1 mg/ml in a 10 mM Tris (pH 7.4) and 0.1 mM EDTA solution made with DEPC-treated water.

- ♦ Use a ribonuclease inhibitor in transcription reactions. The Agilent RNase Block Ribonuclease Inhibitor has been tested and adjusted to work optimally with Agilent's transcription kits.

## **Nonspecific Initiation with T7 and T3 RNA Polymerases**

T7 and T3 RNA polymerases are highly specific for their respective promoters; however, nonspecific initiation of RNA transcripts may occur at the ends of the DNA template. This is most prevalent with a 3'-protruding terminus. Nonspecific initiation may be reduced by increasing the NaCl concentration in the transcription buffers to 100 mM, although this will result in a decrease of the total transcription efficiency by ~50%. When possible, use restriction enzymes that leave blunt or 5'-protruding ends.

If either the T7 or T3 polymerase enzymes are used in molar excess of the DNA template, there is a risk of polymerization from the wrong promoter. T7 polymerase can synthesize RNA inefficiently from a plasmid containing only a T3 promoter. Conversely, T3 polymerase can synthesize RNA inefficiently from a plasmid containing only a T7 promoter. Synthesis is extremely promoter specific when both promoters are present, provided that the enzyme is not in molar excess of the specific promoter. Do not use excessive amounts of the polymerases if promoter specificity is important to the experiment. Best results are obtained when the ratios stated in this instruction manual are followed.

## **Nonradioactive Transcripts**

Nonradioactive transcripts can be used for nucleotide sequencing, *in vitro* translation and injection into cells for *in vivo* translation. Set up the transcription reaction as described, but add 1  $\mu$ l of 10 mM rUTP instead of radioactive rUTP. For larger amounts of RNA, scale up the reaction appropriately. Each molecule of DNA template yields 10–20 nonradioactive RNA molecules if the ribonucleotides are not a limiting factor.

## **DNase Treatment after Transcription**

The DNA template will be present after the transcription reaction and can be removed with RNase-free DNase. After the transcription reaction, add 10 U of RNase-free DNase/ $\mu$ g of DNA template and incubate at 37°C for 15 minutes. Extract with phenol–chloroform [1:1 (v/v)], add a 1/10 volume of 3 M sodium acetate at pH 5.2 and precipitate RNA with 2.5 volumes of 100% (v/v) ethanol.

## High-Specific-Activity RNA Probes

The pBK-CMV phagemid vector can be used to synthesize high-specific-activity, strand-specific RNA probes. The choice between T3 and T7 RNA polymerase promoters will determine which strand will be used as the template.

**Note** *When choosing between T3 and T7 polymerase, it is important to remember that probes used for Northern or S1 analysis must complement the RNA targeted for detection.*

To generate high-specific-activity probes, we suggest using radioactive rATP, rCTP or rUTP as the labeled nucleotide. rGTP should not typically be selected for labeling, since the initiation of RNA transcription requires rGTP, with a  $K_m$  of  $\sim 180$   $\mu\text{M}$ . Thus the concentration of rGTP in the labeling reaction should exceed 180  $\mu\text{M}$ , requiring supplementation of the radioactive rGTP with a high concentration of nonradioactive rGTP, reducing the specific activity of the probe.

Transcription reaction conditions for probe synthesis are provided below. Ribonucleotide composition in this reaction may be adjusted according to the relative importance of achieving full-length and high-quantity of transcript versus high-specific-activity when producing probes. The elongation reaction has a  $K_m$  of 40  $\mu\text{M}$  for each ribonucleotide. Adding 50  $\mu\text{Ci}$  of 500 Ci/mmol [ $^{32}\text{P}$ ]rXTP to a 25- $\mu\text{l}$  reaction produces an rXTP concentration of 4  $\mu\text{M}$ . Any triphosphate present at just 4  $\mu\text{M}$  will not produce many transcripts per template molecule, because the reaction simply runs out of radioactive rXTP. To make large amounts of long radioactive transcripts, supplement the reactions with nonradioactive rXTP.

## Transcription Reactions

1. Add the following components in order:

5  $\mu\text{l}$  of 5 $\times$  transcription buffer<sup>§</sup>  
1  $\mu\text{g}$  of restricted, proteinase K-treated DNA template  
1  $\mu\text{l}$  of 10 mM rATP  
1  $\mu\text{l}$  of 10 mM rCTP  
1  $\mu\text{l}$  of 10 mM rGTP  
1  $\mu\text{l}$  of 1 mM rUTP (optional, see above)  
1  $\mu\text{l}$  of 0.75 M DTT  
1  $\mu\text{l}$  of RNase Block Ribonuclease Inhibitor (optional)  
5  $\mu\text{l}$  of 400–800 Ci/mmol, 10  $\mu\text{Ci}/\mu\text{l}$  [ $\alpha$ - $^{32}\text{P}$ ]rUTP  
10 U of T3 RNA polymerase or T7 RNA polymerase  
DEPC-treated water to a final volume of 25  $\mu\text{l}$

2. Incubate at 37°C for 30 minutes.

<sup>§</sup> See *Preparation of Media and Reagents*.

3. RNA transcripts may be purified away from the unincorporated nucleotides using an RNase-free G-50 column. Take care that there are no ribonucleases present in the column that could degrade the probe.

**Note** *Do not use a large excess of T3 polymerase (10 U per pmol of promoter is sufficient). T3 RNA polymerase may utilize the T7 promoter 1 in 20 times when the T3 enzyme concentration exceeds the T3 promoter concentration by 10-fold. (T3 polymerase in the recommended concentrations will not make T7 transcripts in the presence of a T3 promoter.) If T7 transcripts result from a T3 transcription, decrease the amount of T3 polymerase by 5–10 fold.*

## HYBRIDIZATION CONDITIONS FOR RNA PROBES IN SOUTHERN BLOTS

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**Note** *Prehybridize or hybridize the nitrocellulose membranes using Agilent QuikHyb hybridization solution or follow the procedure outlined below.*

### Prehybridization

**Note** *Prepare and fix the nitrocellulose membranes as outlined in Fixing Replica Sets of Colonies onto Nitrocellulose Membranes.*

1. Prehybridize the membrane with 0.1–0.5 ml/cm<sup>2</sup> of the following prehybridization solution for 2 hours at 42°C with constant agitation in a heat-sealable hybridization bag:

6× SSC buffer  
5× Denhardt's reagent  
20 mM NaH<sub>2</sub>PO<sub>4</sub>  
500 µg/ml denatured, sonicated salmon sperm DNA

### Hybridization and Washes

2. Pour off the prehybridization solution and add the probe to the hybridization bag with the minimum volume of the following hybridization solution:

6× SSC buffer  
20 mM NaH<sub>2</sub>PO<sub>4</sub>  
0.4% (w/v) SDS<sup>¶</sup>  
500 µg/ml denatured, sonicated salmon sperm DNA

3. Incubate overnight at 42°C with constant agitation.
4. Wash twice in a 2× SSC buffer and 0.1% (w/v) SDS wash solution for 15 minutes each at 55°C and twice in a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution for 15 minutes each at 55°C.

<sup>¶</sup> The SDS concentration can be increased to 1% (w/v) for Duralon-UV membranes and Illuminator nylon membranes.

## HYBRIDIZATION CONDITIONS FOR RNA PROBES IN NORTHERN BLOTS

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### Prehybridization

**Note** *Prepare and fix the nitrocellulose membranes as outlined in Fixing Replica Sets of Colonies onto Nitrocellulose Membranes.*

1. Prehybridize the membrane with 0.1–0.5 ml/cm<sup>2</sup> of the following prehybridization solution for ~1 hour at 42°C with constant agitation in a heat-sealable hybridization bag:

50% deionized formamide  
10% dextran sulfate  
1% (w/v) SDS  
1 M NaCl  
100 µg/ml denatured, sonicated salmon sperm DNA

### Hybridization and Washes

2. Pour off the prehybridization solution.
3. Hybridize overnight with ribonucleotide probe at the same temperature and in the same solution as described in step 1 in the previous section (see *Prehybridization*).
4. Wash twice in a 2× SSC buffer and 0.1% (w/v) SDS wash solution for 15 minutes each at 42°C and twice in a 0.1× SSC buffer and 0.1% (w/v) SDS wash solution for 15 minutes each at 42°C.

**Note** *If a high background is observed, increase the temperature or decrease the NaCl concentration for greater stringency.*

## RECOVERY OF SINGLE-STRANDED DNA FROM CELLS CONTAINING THE pBK-CMV PHAGEMID VECTOR

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The pBK-CMV vector is a phagemid that can be secreted as ssDNA in the presence of M13 helper phage. These phagemids contain the IG region of a filamentous f1 phage. This region encodes all the cis-acting functions of the phage required for packaging and replication. In *E. coli* with the F<sup>+</sup> phenotype (containing an F' episome), pBK-CMV phagemid vectors will be secreted as single-stranded f1 "packaged" phage when the bacteria have been infected by a helper phage. Because these filamentous helper phages (M13, f1) will not infect *E. coli* without an F' episome coding for pili, **it is essential to use the XL1-Blue MRF' strain or a similar strain containing the F' episome.<sup>11, 12</sup>**

In the pBK-CMV phagemid vector, the IG region is present in the minus orientation, so that the noncoding strand of the β-galactosidase gene is secreted in the phage particles.

We offer helper phage that *preferentially* package the pBK-CMV phagemid vector. R408 helper phage can be used to produce a large amount of single-stranded pBK-CMV phagemid vector. Typically, 30–50 pBK-CMV molecules are packaged per helper phage DNA molecule. Yields of ssDNA can depend on the specific insert sequence. For most inserts >1 µg of ssDNA can be obtained from a 1.5-ml miniprep if grown in XL1-Blue MRF'. A faint single-stranded helper phage band may appear on a gel at ~4 kb for R408 helper phage. This DNA mixture can be sequenced with primers that are specific for the pBK-CMV phagemid vectors and do not hybridize to the helper phage genome.

**Note** *For excision of the pBK-CMV phagemid vector from the ZAP Express vector, Use the ExAssist interference-resistant helper phage with XLOLR strain.*

## Single-Stranded Rescue Protocol

**Notes** *To complete the single-stranded rescue protocol, it is necessary to use a fresh streak grown on plates containing the appropriate antibiotics or selective growth medium to select for both the F' episome and the phagemid. Grow the pBK-CMV phagemid vector in the XL1-Blue MRF' strain on LB agar plates containing both 12.5 µg/ml of tetracycline and 50 µg/ml of kanamycin.*

*DNA grown in the XL1-Blue MRF' strain must be methylated in an HsdM<sup>+</sup>R<sup>-</sup> strain such as the traditional XL1-Blue strain before being introduced into an HsdR<sup>+</sup> strain (e.g., JM101). Methylation of the DNA by the HsdM strain prevents the DNA from being restricted by the HsdR strain.*

### Day 1

Inoculate a colony from the fresh streak plate into 5 ml of sterile LB broth<sup>§</sup> and grow overnight in the absence of antibiotics.

### Day 2

1. Inoculate 3 ml of 2× YT broth<sup>§</sup> with 300 µl of the fresh overnight culture and grow at 37°C to mid log phase (OD<sub>600</sub> = ~0.3) for 1–2 hours. Grow larger cultures to increase the yield.
2. Add the helper phage to a final concentration of 10<sup>7</sup>–10<sup>8</sup> pfu/ml. Grow the cultures for an additional 8–24 hours at 37°C (with shaking). Transfer 1.5 ml of the infected culture to a microcentrifuge tube.

<sup>§</sup> See *Preparation of Media and Reagents*.

3. Pellet the cells by centrifugation at  $12,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  in a microcentrifuge or other suitable rotor, depending on the volumes used.
4. Transfer 1 ml of the supernatant to a fresh microcentrifuge tube. Add  $\frac{1}{4}$  the volume (250  $\mu\text{l}$ ) of a solution containing 3.5 M ammonium acetate and 20% polyethylene glycol (PEG) to the supernatant and mix well. Incubate at room temperature for 15 minutes to precipitate the phage and phagemid particles.
5. Pellet the phage precipitate by centrifugation for 15 minutes at  $12,000 \times g$  at  $4^{\circ}\text{C}$ . Drain the supernatant well to remove all of the PEG solution. Centrifuge the microcentrifuge tube again for 30 seconds in order to remove any residual supernatant.
6. Resuspend the pellet in 200  $\mu\text{l}$  of TE buffer (see *Preparation of Media and Reagents*) and extract with 100  $\mu\text{l}$  of TE buffer-saturated phenol. This strips the proteins from the ssDNA. Transfer the aqueous phase to a fresh microcentrifuge tube and repeat the extraction with TE buffer-saturated phenol–chloroform. Extract with chloroform and then transfer the supernatant to a fresh microcentrifuge tube.
7. Precipitate the ssDNA from the final aqueous phase by adding a 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% (v/v) ethanol. Mix well and allow the ssDNA to precipitate for 15 minutes at room temperature. Centrifuge at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes.
8. Remove the supernatant. Centrifuge the microcentrifuge tube again for 30 seconds to remove any residual ethanol. Wash the pellet with 200  $\mu\text{l}$  of 70% (v/v) ethanol. Resuspend the pellet, containing 1–5  $\mu\text{g}$  of ssDNA/ml of initial phage supernatant, in 20  $\mu\text{l}$  of TE buffer and store at  $4^{\circ}\text{C}$ .
9. The ssDNA can be visualized on an ethidium bromide (EtBr)-stained 1% (w/v) agarose gel. Single-stranded pBK-CMV (without insert) runs at  $\sim$ 1.4 kb relative to dsDNA standards on agarose gels run in 20 mM Tris-acetate (pH 8.0) and 1 mM EDTA.

## SITE-DIRECTED MUTAGENESIS

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The ssDNA isolated according to the *Single-Stranded Rescue Protocol* can be used for site-directed oligonucleotide mutagenesis. The following protocol is recommended.

### Kinase Reaction for the Oligonucleotide

(Use 100 ng of the oligonucleotide in a 40- $\mu$ l reaction.)

100 ng of the oligonucleotide  
4  $\mu$ l of 10 $\times$  ligase buffer  
4  $\mu$ l of 10 mM rATP  
2  $\mu$ l of T4 PNK (10 U)  
Water to 40  $\mu$ l

Incubate at 37°C for 30 minutes.

### Synthesis of the Mutant DNA Strand

1. Anneal the oligonucleotide by combining the following components:

20  $\mu$ l (50 ng) of the oligonucleotide from the above kinase reaction  
5  $\mu$ l of ssDNA (1  $\mu$ g of template)

Incubate at 65°C for 10 minutes and then incubate at room temperature for 5 minutes.

2. Perform the primer extension reaction by adding the following to the annealing reaction:

4  $\mu$ l of 10 $\times$  ligase buffer  
2  $\mu$ l of 2.5 mM dNTPs (N = A, C, G and T in equal concentrations)  
4  $\mu$ l of 10 mM rATP  
1  $\mu$ g of ssDNA binding protein  
 $X$   $\mu$ l of water  
1.5 U of DNA polymerase I (Klenow fragment)  
4 Weiss U of T4 DNA ligase  
40  $\mu$ l total reaction volume

Incubate at room temperature for 3–4 hours.

3. Transform the *E. coli* strain XL1-Blue MRF' with 10  $\mu$ l of synthesis reaction and plate onto nitrocellulose membranes across three plates.
4. Screen colonies as described in *Colony Screening by Hybridization* (1% mutants should be obtained).

## PLASMID BOILING MINIPREP PROTOCOL

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The following protocol yields high-quality dsDNA template simply and rapidly.<sup>13</sup> (Caution: *Escherichia coli* strain HB101 and derivatives give low yields using this protocol.) This DNA is suitable for restriction enzyme digestion or for enzyme sequencing.

1. Grow a 3-ml culture overnight in LB broth plus kanamycin (50 µg/ml) from a single colony.
2. Pellet 1.5 ml of the culture in a microcentrifuge at 4°C for 2 minutes. Remove the supernatant by aspiration.
3. Resuspend the pellet in 110 µl of STETL buffer (see *Preparation of Media and Reagents*).
4. Place the tube in a boiling water bath for 30 seconds.
5. Immediately spin the tube in a microcentrifuge at 4°C for 15 minutes.
6. Remove and discard the pellet with a sterile toothpick. Save the supernatant. [RNase treatment (20 µg/ml) is optional at this stage.]
7. Add 110 µl of isopropanol to the supernatant and immediately spin the tube in a microcentrifuge for 15 minutes.
8. Resuspend the pellet in 100 µl of TE buffer.
9. Extract twice with an equal volume of phenol–chloroform [1:1 (v/v)] and once with chloroform.

**Note** *To purify the sample, StrataClean resin may be used in place of the phenol–chloroform extractions.*

10. Add an equal volume of 7.5 M ammonium acetate and precipitate with 2.5 volumes of ethanol. Incubate on ice for 15 minutes and spin at 4°C for 20 minutes.
11. Rinse with 1 ml of 80% (v/v) ethanol and spin in a microcentrifuge for 1 minute.
12. Vacuum dry the pellets.
13. Resuspend the pellets in 15 µl of TE buffer.
14. Use 5 µl of this DNA (about 2.0 µg) for sequencing.

## TROUBLESHOOTING

Observation	Suggestions
Low or no expression in eukaryotic cells	<p>Remove the prokaryotic sequences from the transcript by digesting the plasmid with <i>Nhe</i> I and <i>Spe</i> I, religating, transforming and checking that the <i>lac</i> promoter and <i>lacZ</i> ATG (202 bp) were removed. Other sites may be used if these enzymes cut within the insert. This will aid expression if any of the following are true.</p> <ol style="list-style-type: none"><li>1. Interference of expression by upstream prokaryotic sequences in the 5' UT region of the eukaryotic transcript</li><li>2. Decreased specific activity or inappropriate folding due to expression of the fusion protein</li><li>3. Interference of translation initiation due to the presence of two translation initiation sites (<i>lacZ</i> ATG and insert ATG)</li></ol>
	Insure that the insert sequence and the CMV promoter are not methylated. If so, grow the tissue culture cells in the presence of 5-azacytidine to prevent methylation <sup>14</sup>

## PREPARATION OF MEDIA AND REAGENTS

<b>LB Broth (per Liter)</b> 10 g of NaCl 10 g of tryptone 5 g of yeast extract Adjust to pH 7.0 with 5 N NaOH Add deionized H <sub>2</sub> O to a final volume of 1 liter Autoclave	<b>LB Agar (per Liter)</b> 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Adjust pH to 7.0 with 5 N NaOH Add deionized H <sub>2</sub> O to a final volume of 1 liter Autoclave Pour into petri dishes (~25 ml/100-mm plate)
<b>LB-Tetracycline Broth (per Liter)</b> 1 liter of LB broth Autoclave Cool to 55°C Add 12.5 mg of filter-sterilized tetracycline Store broth in a dark, cool place as tetracycline is light-sensitive	<b>LB-Tetracycline Agar (per Liter)</b> 1 liter of LB agar Autoclave Cool to 55°C Add 12.5 mg of filter-sterilized tetracycline Pour into petri dishes (~25 ml/100-mm plate) Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods as tetracycline is light-sensitive
<b>LB-Kanamycin Broth (per Liter)</b> 1 liter of LB broth Autoclave Cool to 55°C Add 50 mg of filter-sterilized kanamycin	<b>LB-Kanamycin Agar (per Liter)</b> 1 liter of LB agar Autoclave Cool to 55°C Add 50 mg of filter-sterilized kanamycin Pour into petri dishes (~25 ml/100-mm plate)
<b>LB-Tetracycline-Kanamycin Agar (per Liter)</b> 1 liter of LB agar Autoclave Cool to 55°C Add 12.5 mg of filter-sterilized tetracycline Add 50 mg of filter-sterilized kanamycin Pour into petri dishes (~25 ml/100-mm plate) Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods as tetracycline is light-sensitive	<b>LB-Chloramphenicol Agar (per Liter)</b> 1 liter of LB agar Autoclave Cool to 55°C Add 30 mg of filter-sterilized chloramphenicol Pour into petri dishes (~25 ml/100-mm plate)

<p><b>M9 Media (per Liter)</b></p> <p>6.0 g of dibasic sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>)  3.0 g of monobasic potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>)  1.0 g of ammonium chloride (NH<sub>4</sub>Cl)  0.5 g of NaCl  Water to 1 liter  Autoclave  While autoclaving, make the following solution:  1.0 ml of 1 M MgSO<sub>4</sub>  2.0 g of glucose  0.1 ml of 1 M CaCl<sub>2</sub>  1.0 ml of 1 M thiamine-HCl  Water to 10 ml and filter sterilize</p> <p>Add the above solution to the cooled M9 media</p>	<p><b>STETL Buffer</b></p> <p>8.0% sucrose  0.5% Triton® X-100  50.0 mM Tris (pH 8.0)  50.0 mM EDTA  0.5 mg/ml lysozyme  All components except lysozyme can be prepared and stored indefinitely at 4°C. The lysozyme is made as a 5 mg/ml stock and is stored in small aliquots at -20°C. Do not reuse the lysozyme stock after thawing.</p> <p><b>1 × STE Buffer</b></p> <p>100 mM NaCl  20 mM Tris-HCl (pH 7.5)  10 mM EDTA</p>
<p><b>20× SSC Buffer (per Liter)</b></p> <p>175.3 g of NaCl  88.2 g of sodium citrate  800.0 ml of water  10.0 N NaOH  Adjust to pH 7.0 with a few drops of a 10.0 N NaOH  Adjust volume to 1 liter with water</p>	<p><b>50× Denhardt's Reagent (per 500 ml)</b></p> <p>5 g of Ficoll  5 g of polyvinylpyrrolidone  5 g of BSA (Fraction V)  Water to 500 ml  Filter through a disposable filter  Dispense into aliquots and store at -20°C</p>
<p><b>10× PIPES Buffer</b></p> <p>4.0 M NaCl  0.1 M PIPES buffer (pH 6.5)</p>	<p><b>5× Transcription Buffer</b></p> <p>200 mM Tris-HCl (pH 8.0)  40 mM MgCl<sub>2</sub>  10 mM spermidine  250 mM NaCl</p>
<p><b>2× YT Broth (per Liter)</b></p> <p>10 g of NaCl  10 g of yeast extract  16 g of tryptone  Adjust to pH 7.5 with NaOH</p>	<p><b>TE Buffer</b></p> <p>10 mM Tris-HCl (pH 7.5)  1 mM EDTA</p>
<p><b>Optimal Buffer #7</b></p> <p>1.5 M NaCl  100 mM Tris-HCl (pH 7.7)  70 mM MgCl<sub>2</sub>  10 mM DTT  100 µg/ml of bovine serum albumin (BSA)</p>	<p><b>10× Ligase Buffer</b></p> <p>500 mM Tris-HCl (pH 7.5)  70 mM MgCl<sub>2</sub>  10 mM DTT</p> <p><b>Note</b> rATP is added separately in the ligation reaction.</p>

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## ENDNOTES

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